Effect of Acclimation Conditions on 3D Human *in vitro* ALI Airway Models: Exposure-induced Inflammatory Response and Sampling Location-specific Results

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ABSTRACT

Reconstructed human airway (RHuA) tissues are 3D organotypic models that feature relevant cell types and more accurately reflect the pulmonary airway *in vivo*. We conducted a pilot study to characterize and test acclimation conditions on RHuA tissue inflammatory responses. MatTek EpiAirway™ tissues were received and acclimated for 24 or 48 hr in medium ± 2 μM hydrocortisone (HC). Triplicate tissues were treated apically with 15 μg/mL Poly I:C or 5 μg/mL lipopolysaccharide for 24 hr as reference agents. Apical rinse, tissue lysate, and basal medium samples were collected. Total protein, LDH, IL-6, IL-8, and IP-10 were assayed in single-plex, followed by a 30-plex human cytokine evaluation. Single-plex results from the 48 hr HC(+) acclimation indicated a 6-fold greater average biomarker increase compared with 24hr HC(+) or HC(-) groups following challenge. Markers were up to ~5-fold higher in apical rinse and tissue lysates compared to basal medium. IP-10 exhibited the greatest increase over control (160-fold in tissue lysate; 139-fold in apical rinse, and 25-fold in basal medium) following Poly I:C challenge. The 30-plex assay verified and expanded the quantitation of inflammatory responses. Both reference agents elicited distinct proinflammatory, cytokine, and chemokine marker profiles that defined a material-specific "signature" of inflammation. Optimized tissue acclimation and appropriate sampling from various RHuA compartments following apical exposures should be considered during study design. RHuA are increasingly used to assess inhalation exposures, such as cigarette smoke and E-cigarette vapors. Characterizing multiple sample compartments following apical challenge will identify exposure specific inflammatory responses. These responses may offer increased sensitivity for comparisons of cigarettes and novel products such as E-cigarettes.
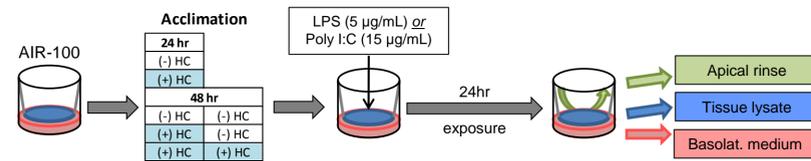
INTRODUCTION

With the emergence of modern 3D tissue culture systems, the research community now has capabilities that far exceed cell culture technology first developed in the mid-20th century. Human reconstructed airway tissues (RHuA) consist of heterogeneous cell types, pseudostratified into layers and resemble airway structures found *in vivo*. These RHuA, upon challenge, are well-known to produce immune-mediator responses, demonstrate functional changes, allow non-invasive tight-junction barrier analysis, as well as canonical cytotoxicity and viability measurements. Cell specific assessments such as Goblet cell-based mucin production, columnar cell changes in ciliary beat frequency, and changes in basal cells are possible. These markers can contribute to the assessment of more complex-tissue specific changes that may correspond to key events that may lead to advanced pulmonary disease states such as chronic-obstructive pulmonary disease (COPD).

The dynamic properties of these 3D RHuA tissues have prompted us to evaluate differences in acclimation conditions, and how these tissues will respond in a spatially relevant manner upon challenge with two known respiratory toxicants, lipopolysaccharide (LPS) and a viral mimetic, Polyinosinic:polycytidylic acid (Poly I:C). The compartment-based novelty of these RHuA allows us to better understand response localization (inflammatory mediators and the leakage marker LDH) and assess spatially relevant changes in a manner that may reflect tissue responses *in vivo*.

MATERIALS & METHODS

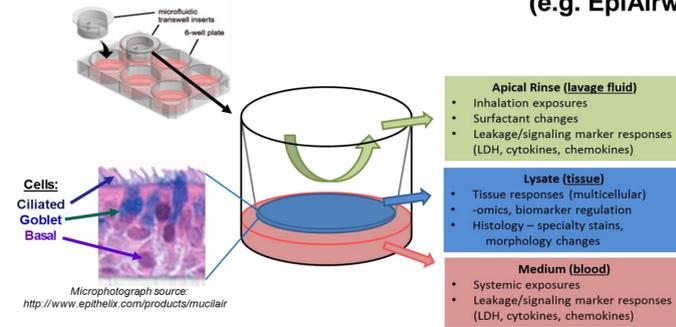3D RHuA tissues: MatTek EpiAirway™ AIR-100

- Upon receipt, tissues were acclimated for 24 or 48 hr using the manufacturers medium (AIR-100 ASY), or spiked with 2 μM hydrocortisone (HC) and placed into culture (37° C in a humidified incubator)

- Following acclimation, tissue inserts were placed into new plates containing fresh medium, without HC and the apical surface of each tissue was rinsed, prior to apical exposure (100 μL DPBS vehicle ± treatment).

- Apical rinses, medium, and tissue lysates were collected and fresh aliquots assayed for LDH content (G-Biosciences, Cat. # 786-210), or frozen for subsequent cytokine analysis

- Initial cytokines (IL-6, IL-8, and IP-10) were assayed by ELISA (R&D Systems, ELISA kits for IL-6, Il-8, IP-10)

- All absorbance values were read on a MDS Vmax instrument

- Subsequent, wide-array cytokine analysis for proinflammatory, cytokine, and chemokine panels were assayed using the MSD Sector 6000 (Cat. # 786-210) with the V-PLEX Human Cytokine 30-Plex Kit (Cat #K15054D-1)

In Vitro Platform

3D Pulmonary Model: Human Reconstructed Airway (RHuA) Model
(e.g. EpiAirway™ or MucilAir™)

Cells: Ciliated, Goblet, Basal

Microphotograph source: http://www.epithelix.com/products/mucilair

Benefit of "Compartments"

Apical Rinse (lavage fluid)
- Inhalation exposures
- Surfactant changes
- Leakage/signaling marker responses (LDH, cytokines, chemokines)

Lysate (tissue)
- Tissue responses (multicellular)
- -omics, biomarker regulation
- Histology – specialty stains, morphology changes

Medium (blood)
- Systemic exposures
- Leakage/signaling marker responses (LDH, cytokines, chemokines)
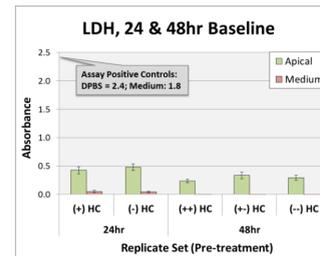
- 3D RHuA tissues offer 3 different sampling points that collectively, could reflect marker responses reflective to in lavage fluid, the tissue itself, and systemic markers that may be found in the circulation
- Pseudostratification results in a heterogeneous cell types located in different locations
- Non-invasive time point collections (apical rinse, TEER, medium) allow monitoring the progression of events
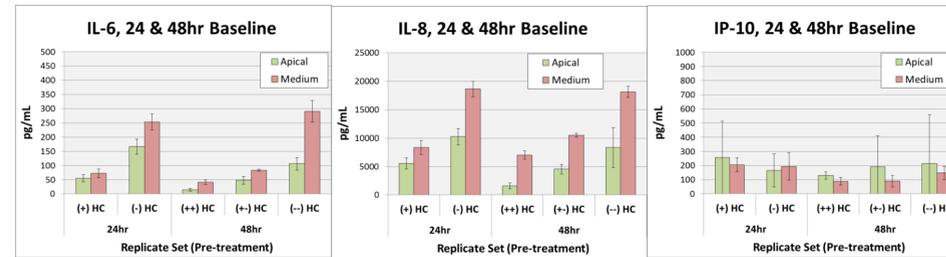
RESULTS

Effect of Acclimation Conditions: Baseline Values*

LDH, 24 & 48hr Baseline

Assay Positive Controls: DPBS = 2.4; Medium: 1.8

LDH Release

- Acclimation conditions have minimal effect on LDH release in both apical and basal compartments
- After 48hr acclimation, regardless of HC inclusion, medium levels of LDH activity are not detectable
- Modest amounts of LDH content in the apical rinse may be impacted by cells sloughing during wash step

* Treatment groups were pooled, based on acclimation paradigm. 24 hr baseline values were derived from either 24 or 36 tissues while 48hr acclimation baseline values were derived from 12 tissues.

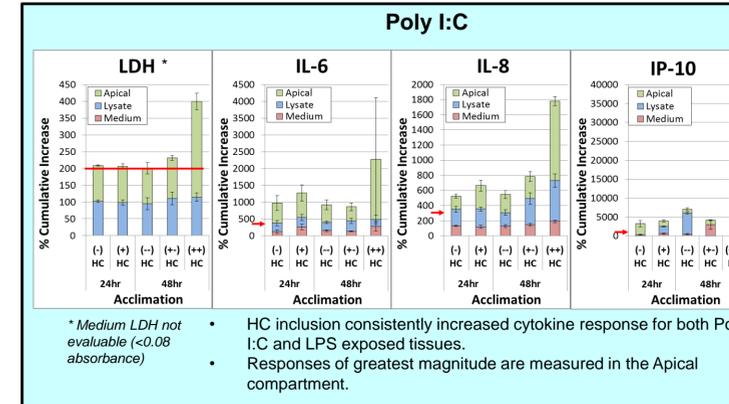IL-6, 24 & 48hr Baseline   IL-8, 24 & 48hr Baseline   IP-10, 24 & 48hr Baseline
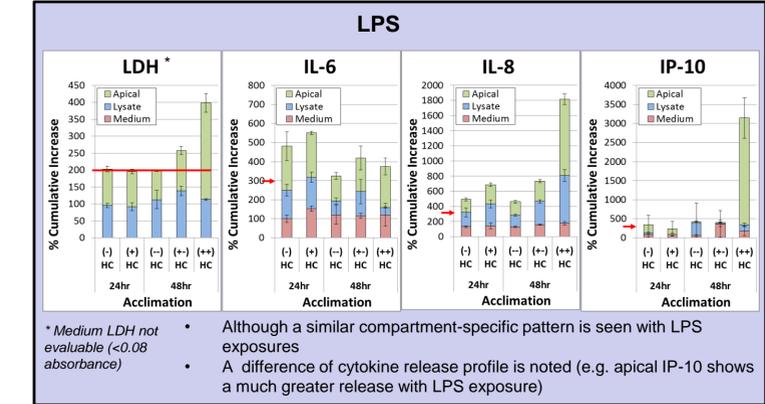
Cytokine Release

- HC inclusion results in a lower baseline release of IL-6 and IL-8 in both Apical and Basolateral compartments, at both 24hr and 48hr acclimation periods.
- The fold reduction of baseline IL-6 was up to ~12-fold and for IL-8 was up to ~6-fold. The greatest differences were seen between 48hr (++) HC vs 24hr (-) HC).
- IP-10 levels detected in the Apical rinse and medium were low and did not demonstrate the same differences

RESULTS cont.

Effect of Acclimation Conditions: Response to Toxicants

Poly I:C

LDH *   IL-6   IL-8   IP-10

* Medium LDH not evaluable (<0.08 absorbance)

- HC inclusion consistently increased cytokine response for both Poly I:C and LPS exposed tissues.
- Responses of greatest magnitude are measured in the Apical compartment.

LPS

LDH *   IL-6   IL-8   IP-10

* Medium LDH not evaluable (<0.08 absorbance)

- Although a similar compartment-specific pattern is seen with LPS exposures
- A difference of cytokine release profile is noted (e.g. apical IP-10 shows a much greater release with LPS exposure)
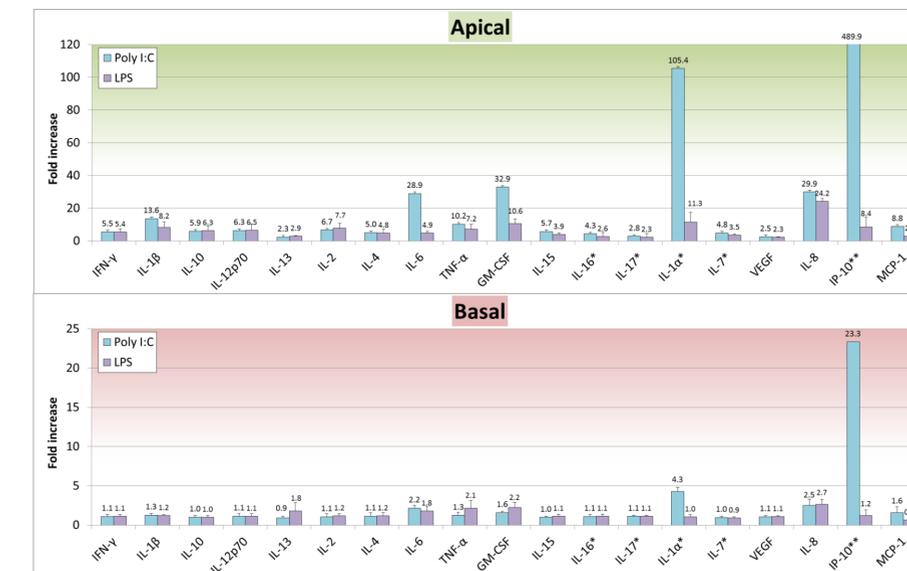
Optimized Acclimation Condition (++) HC: 30-plex Response to Toxicants

Table: Ratio of Increase (Apical Rinse/Basolateral Medium)

Proinflammatory	IFN-γ	IL-1b	IL-10	IL-12p70	IL-13	IL-2	IL-4	IL-6	TNF-α
Poly(I:C) HC48+	5.0	10.6	5.9	5.6	2.5	6.3	4.4	13.4	8.1
LPS HC48+	4.8	6.8	6.3	5.7	1.6	6.6	4.1	2.7	3.4
Cytokine	GM-CSF	IL-12p40	IL-15	IL-16	IL-17	IL-1α	IL-5	IL-7	TNF-β	VEGF
Poly(I:C) HC48+	20.9	NE	5.7	4.0	2.5	24.7	NE	5.0	NE	2.4
LPS HC48+	4.8	NE	3.5	2.3	2.1	10.9	NE	3.9	NE	2.2
Chemokine	Eotaxin	Eotaxin-3	IL-8	IP-10	MCP-1	MCP-4	MDC	MIP-1α	MIP-1β	TARC
Poly(I:C) HC48+	NE	NE	11.9	21.0	5.6	NE	NE	NE	NE	NE
LPS HC48+	NE	NE	9.1	6.8	4.5	NE	NE	NE	NE	NE

NE: not evaluable

Increases: Apical vs Basolateral Ratios

- All markers demonstrated greater increase in the apical compartment where exposures occurred
- Ratios varied substantially and were marker dependent

Poly I:C vs LPS Marker Responses: Wide Panel Array

Apical

Basal

* marker values are near or below limits of quantitation   ** one or more values of group are > limits of quantitation

- Changes, including exposure-specific responses, differ based on sampling location
- Increases of markers are apparent in the apical compartment, but may measure as equivalent to negative control

CONCLUSIONS

1. This pilot study has elucidated aspects of RHuA evaluation that require consideration when assessing exposure-induced biomarker changes

2. Using the optimal acclimation paradigm may allow for a greater signal following RHuA exposure to the apical (airway) compartment

3. Sampling in a second physiologically-relevant location (apical compartment) location will highlight changes in cytotoxicity, viability, and in mediators of inflammation not detected in medium

4. The use of a wide marker array can show exposure material-specific patterns of change – including those involved in key events that may help assess pathways leading to COPD

5. Additional studies may determine that material-specific patterns of change may deliver "signature" responses that will allow distinguish similar products' comparative risk, such as modified risk tobacco products or other inhaled materials

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